HUMAN ANTIBODIES DERIVED FROM IMMUNIZED XENOMICE

Technical Field

The invention relates to the field of
immunology, and in particular to the production of
5 antibodies. More specifically, it concerns producing such
antibodies by a process which includes the step of
immunizing a transgenic animal with an antigen to which
antibodies are desired. The transgenic animal has been
modified so as to produce human, as opposed to endogenous
10 antibodies.

Background Art

PCT application WO 94/02602, published 3
February 1994 and incorporated herein by reference,
describes in detail the production of transgenic nonhuman
15 animals which are modified so as to produce antibodies
with fully human variable regions rather than endogenous
antibodies in response to antigenic challenge. Briefly,
the endogenous loci encoding the light and heavy
immunoglobulin chains are incapacitated in the transgenic
20 hosts and loci encoding human heavy and light chain
proteins are inserted into the genome. In general, the
animal which provides all the desired modifications is
obtained by cross-breeding intermediate animals containing
fewer than the full complement of modifications. The
25 preferred embodiment of the nonhuman animal described in
the specification is a mouse. Thus, mice, specifically,
are described which, when administered immunogens, produce
antibodies with human variable regions, including fully

human antibodies, rather than murine antibodies that are immunospecific for these antigens.

The availability of such transgenic animals makes possible new approaches to the production of fully
5 human antibodies. Antibodies with various immunospecificities are desirable for therapeutic and diagnostic use. Those antibodies intended for human therapeutic and *in vivo* diagnostic use, in particular, have been problematic because prior art sources for such
10 antibodies resulted in immunoglobulins bearing the characteristic structures of antibodies produced by nonhuman hosts. Such antibodies tend to be immunogenic when used in humans.

The availability of the nonhuman, immunogen-
15 *responsive transgenic animals described in the above-* referenced WO 94/02602 make possible convenient production of human antibodies without the necessity of employing human hosts.

Disclosure of the Invention

20   The invention is directed to methods to produce human antibodies by a process wherein at least one step of the process includes immunizing a transgenic nonhuman animal with the desired antigen. The modified animal fails to produce endogenous antibodies, but instead
25 produces B-cells which secrete immunoglobulins with fully human variable regions. The antibodies produced include fully human antibodies and can be obtained from the animal directly, or from immortalized B-cells derived from the animal. Alternatively, the genes encoding the
30 immunoglobulins with human variable regions can be

recovered and expressed to obtain the antibodies directly or modified to obtain analogs of antibodies such as, for example, single chain F_v molecules.

Thus, in one aspect, the invention is directed
5 to a method to produce an immunoglobulin with a fully human variable region to a specific antigen or to produce an analog of said immunoglobulin by a process which comprises immunizing a nonhuman animal with the antigen under conditions that stimulate an immune response. The
10 nonhuman animal is characterized by being substantially incapable of producing endogenous heavy or light immunoglobulin chain, but capable of producing immunoglobulins either with both human variable regions and constant regions or with fully human variable regions
15 or both. In the resulting immune response, the animal produces B cells which secrete immunoglobulins, with at least variable regions that are fully human, specific for the antigen. The human immunoglobulin of desired specificity can be directly recovered from the animal, for
20 example, from the serum, or primary B cells can be obtained from the animal and immortalized. The immortalized B cells can be used directly as the source of human antibodies or, alternatively, the genes encoding the antibodies can be prepared from the immortalized B cells
25 or from primary B cells of the blood or lymphoid tissue (spleen, tonsils, lymph nodes, bone marrow) of the immunized animal and expressed in recombinant hosts, with or without modification, to produce the immunoglobulin or its analogs. In addition, the genes encoding the
30 repertoire of immunoglobulins produced by the immunized animal can be used to generate a library of

immunoglobulins to permit screening for those variable regions which provide the desired affinity. Clones from the library which have the desired characteristics can then be used as a source of nucleotide sequences encoding

5 the desired variable regions for further manipulation to generate antibodies or analogs with these characteristics using standard recombinant techniques.

In another aspect, the invention relates to an immortalized nonhuman B cell line derived from the above

10 described animal. In still another aspect, the invention is directed to a recombinant host cell which is modified to contain the gene encoding either the human immunoglobulin with the desired specificity, or an analog thereof which exhibits the same specificity.

15 In still other aspects, the invention is directed to antibodies or antibody analogs prepared by the above described methods and to recombinant materials for their production.

In still other aspects, the invention is

20 directed to antibodies with fully human variable regions, including fully human antibodies which are immunospecific with respect to particular antigens set forth herein and to analogs which are similarly immunospecific, as well as to the recombinant materials useful in the production of

25 these antibodies.

Brief Description of the Drawings

Figure 1 shows the serum titers of anti-IL-6 antibodies from a xenomouse immunized with human IL-6 and which antibodies contain human kappa light chains and/or

30 human μ heavy chains.

Figure 2 shows the serum titers of anti-IL-8 antibodies from a xenomouse immunized with human IL-8 and which antibodies contain human kappa light chains and/or human μ heavy chains.

5   Figure 3 shows the serum titers of anti-TNFα antibodies from a xenomouse immunized with human TNF-α and which antibodies contain human kappa light chains and/or human μ heavy chains.

Figure 4 shows the serum titers of anti-CD4

10 antibodies from a xenomouse immunized with human CD4 and which antibodies contain human kappa light chains and/or human μ heavy chains.

Figure 5 shows the serum titers of a xenomouse immunized with 300.19 cells expressing L-selectin at their

15 surface. In the ELISA assay used, these antibodies are detectable only if they carry human μ constant region heavy chains.

Figure 6 shows the serum titers of a xenomouse immunized with 300.19 cells expressing L-selectin at their

20 surface. In the ELISA assay used, these antibodies are detectable only if they carry human kappa light chains.

Figure 7 shows the serum titers of a xenomouse immunized with 300.19 cells expressing L-selectin. In this ELISA, these antibodies are detectable if they carry

25 human kappa light chain and/or murine γ constant regions.

Figure 8 shows a FACS analysis of human neutrophils coupled to sera from a xenomouse (A195-2) immunized with human L-selectin and labeled with an antibody immunoreactive with murine heavy chain γ constant

30 region.

Figure 9 shows a FACS analysis of human neutrophils incubated with serum from a xenomouse (A195-2) immunized with human L-selectin and labeled with an antibody immunoreactive with human light chain kappa

5   region.

Figure 10 is a diagram of a plasmid used to transfect mammalian cells to effect the production of the human protein gp39.

Figure 11 represents the serum titration curve

10   of mice immunized with CHO cells expressing human gp39. The antibodies detected in this ELISA must be immunoreactive with gp39 and contain human heavy chain μ constant regions or human kappa light chains.

Figure 12 shows the results of a FACS analysis

15   of antibodies from a xenomouse (labeled A247-4) immunized with human gp39 reacted with activated human T cells. Figure 12A shows the separation of human activated T cells into CD4+ and CD4- populations. Panel B shows the results of a FACS analysis of the activated CD4+ T cells with

20   antibodies from the xenomouse immunized with gp39 which contain murine heavy chain γ constant regions; panel C shows the corresponding results with respect to CD4- populations.

Figure 13 is a titration curve with respect to

25   monoclonal antibodies secreted by the hybridoma clone D5.1. This clone is obtained from a xenomouse immunized with tetanus toxin C (TTC) and contains human kappa light chain and human μ constant region in the heavy chain.

Figure 14 is a titration curve with respect to

30   the hybridoma supernatant from clone K4.1. This hybridoma clone is obtained from a xenomouse immunized with TTC and

contains human kappa light chain and heavy chain having
the murine γ constant region.

Figure 15 shows binding curves for various
concentrations of the K4.1 monoclonal antibody in a
5   determination of the affinity of the monoclonal with its
antigen in a BIAcore instrument.

Figure 16 shows the complete nucleotide sequence
of the heavy chain from the antibody secreted by K4.1.

Figure 17 shows the complete nucleotide sequence
10 of the light chain from the antibody secreted by K4.1.

Figure 18 shows the complete nucleotide sequence
of the heavy chain from the antibody secreted by D5.1.

Figure 19 shows the complete nucleotide sequence
of the light chain from the antibody secreted by D5.1.

15 Modes of Carrying Out the Invention

In general, the methods of the invention include
administering an antigen for which human forms of
immunospecific reagents are desired to a transgenic
nonhuman animal which has been modified genetically so as
20 to be capable of producing human, but not endogenous,
antibodies. Typically, the animal has been modified to
disable the endogenous heavy and/or light chain loci in
its genome, so that these endogenous loci are incapable of
the rearrangement required to generate genes encoding
25 immunoglobulins in response to an antigen. In addition,
the animal will have been provided, stably, in its genome,
at least one human heavy chain locus and at least one
human light chain locus so that in response to an
administered antigen, the human loci can rearrange to

provide genes encoding human immunoglobulins
immunospecific for the antigen.

The details for constructing such an animal
useful in the method of the invention are provided in the
5   PCT application WO 94/02602 referenced above.

For production of the desired antibodies, the
first step is administration of the antigen. Techniques
for such administration are conventional and involve
suitable immunization protocols and formulations which
10 will depend on the nature of the antigen per se. It may
be necessary to provide the antigen with a carrier to
enhance its immunogenicity and/or to include formulations
which contain adjuvants and/or to administer multiple
injections, and the like. Such techniques are standard
15 and optimization of them will depend on the
characteristics of the particular antigen for which
immunospecific reagents are desired.

As used herein, the term "immunospecific
reagents" includes immunoglobulins and their analogs. The
20 term "analogs" has a specific meaning in this context. It
refers to moieties that contain the fully human portions
of the immunoglobulin which account for its
immunospecificity. In particular, variable regions
including the complementarity determining regions (CDRs)
25 are required, along with sufficient portions of the
framework regions (FRs) to result in the appropriate three
dimensional conformation. Typical immunospecific analogs
of antibodies include $F_{(ab')_2}$, $F_{ab'}$, and F_{ab} regions.
Modified forms of the variable regions to obtain, for
30 example, single chain F_V analogs with the appropriate
immunospecificity are known. A review of such F_V

construction is found, for example, in Tibtech (1991)
9:____. The construction of antibody analogs with
multiple immunospecificities is also possible by coupling
the human variable regions derived from antibodies with
5 varying specificities.

The variable regions with fully human
characteristics can also be coupled to a variety of
additional substances which can provide toxicity,
biological functionality, alternative binding
10 specificities and the like. The moieties including the
fully human variable regions produced by the methods of
the invention include single-chain fusion proteins,
molecules coupled by covalent methods other than those
involving peptide linkages, and aggregated molecules.
15 Examples of analogs which include variable regions coupled
to additional molecules covalently or noncovalently
include those in the following nonlimiting illustrative
list. Traunecker, A. et al. Int J Cancer Supp (1992) Supp
7:51-52 describe the bispecific reagent janusin in which
20 the F_V region directed to CD3 is coupled to soluble CD4 or
to other ligands such as OVCA and IL-7. Similarly, the
fully human variable regions produced by the method of the
invention can be constructed into F_V molecules and coupled
to alternative ligands such as those illustrated in the
25 cited article. Higgins, P.J. et al. J Infect Disease
(1992) 166:198-202 describe a heteroconjugate antibody
composed of OKT3 cross-linked to an antibody directed to a
specific sequence in the V3 region or GP120. Such
heteroconjugate antibodies can also be constructed using
30 at least the human variable regions contained in the
immunoglobulins produced by the invention methods.

Additional examples of bispecific antibodies include those
described by Fanger, M.W. et al. Cancer Treat Res (1993)
68:181-194 and by Fanger, M.W. et al. Crit Rev Immunol
(1992) 12:101-124. Conjugates that are immunotoxins

5 including conventional antibodies have been widely
described in the art. The toxins may be coupled to the
antibodies by conventional coupling techniques or
immunotoxins containing protein toxin portions can be
produced as fusion proteins. The analogs of the present

10 invention can be used in a corresponding way to obtain
such immunotoxins. Illustrative of such immunotoxins are
those described by Byrs, B.S. et al. Seminars Cell Biol
(1991) 2:59-70 and by Fanger, M.W. et al. Immunol Today
(1991) 12:51-54.

15 In short, the genes encoding the immunoglobulins
produced by the transgenic animals of the invention can be
retrieved and the nucleotide sequences encoding the fully
human variable region can be manipulated according to
known techniques to provide a variety of analogs such as

20 those described above. In addition, the immunoglobulins
themselves containing the human variable regions can be
modified using standard coupling techniques to provide
conjugates retaining immunospecificity and fully human
characteristics in the immunospecific region.

25 Thus, immunoglobulin "analogs" refers to
moieties which contain those portions of the antibodies of
the invention which retain their human characteristics and
their immunospecificity. These will retain sufficient
human variable region to provide the desired specificity.

30 As stated above, all of the methods of the
invention include administering the appropriate antigen to

the transgenic animal. The recovery or production of the antibodies themselves can be achieved in various ways.

First, and most straightforward, the polyclonal antibodies produced by the animal and secreted into the

5   bloodstream can be recovered using known techniques. Purified forms of these antibodies can, of course, be readily prepared by standard purification techniques, preferably including affinity chromatography with respect to the particular antigen, or even with respect to the

10   particular epitope of the antigen for which specificity is desired. In any case, in order to monitor the success of immunization, the antibody levels with respect to the antigen in serum will be monitored using standard techniques such as ELISA, RIA and the like.

15   It will be noted, from the examples below, that a portion of the polyclonal antiserum obtained may include an endogenous heavy chain constant region derived from the host, even though the variable regions are fully human. Under these circumstances, to the extent that an

20   application requires fully human antibodies, use of the polyclonal antiserum directly would be inappropriate. However, the presence of these chimeras, which is believed to result from in vivo isotype switching as described by Gerstein et al. Cell (1990) 63:537, is not problematic, in

25   view of conventional purification and modification methods and in view of the availability of alternative methods to recover fully human antibodies, if desired, described in the following paragraphs.

First, and most simply, the polyclonal antiserum

30   could be subjected to suitable separation techniques to provide compositions containing only fully human

immunoglobulins. Portions of the serum which display
characteristics of the host species can be removed, for
example, using affinity reagents with the appropriate anti
species immunoglobulins or immunospecific portions
5   thereof. Furthermore, for applications where only the
variable regions of the antibodies are required, treating
the polyclonal antiserum with suitable reagents so as to
generate F_{ab}, $F_{ab'}$, or $F_{(ab')_2}$ portions results in
compositions containing fully human characteristics. Such
10   fragments are sufficient for use, for example, in
immunodiagnostic procedures involving coupling the
immunospecific portions of immunoglobulins to detecting
reagents such as radioisotopes. Thus, for some
applications, the polyclonal antiserum can be treated to
15   provide compositions with the desired characteristics
including compositions consisting essentially of fully
human antibodies and compositions including immunoglobulin
analogs wherein the immunospecific portion is fully human.

         Alternatively, immunoglobulins and analogs with
20   desired characteristics can be generated from immortalized
B cells derived from the transgenic animals used in the
method of the invention or from the rearranged genes
provided by these animals in response to immunization. It
will be apparent that hybridomas derived from the B cells
25   of the immunized animal can be screened so as to choose
only those secreting fully human antibodies and that the
genetic material can be recovered from the hybridomas or
from lymphocytes in spleen, blood, or lymph nodes of the
immunized animal and manipulated using conventional
30   techniques to replace any endogenous constant region with
a human one or to produce a desired analog.

     Thus, as an alternative to harvesting the
antibodies directly from the animal, the B cells can be
obtained, typically from the spleen, but also, if desired,
from the peripheral blood lymphocytes or lymph nodes and
5   immortalized using any of a variety of techniques, most
commonly using the fusion methods described by Kohler and
Milstein. The resulting hybridomas (or otherwise
immortalized B cells) can then be cultured as single
colonies and screened for secretion of antibodies of the
10   desired specificity. As described above, the screen can
also include a determination of the fully human character
of the antibody. For example, as described in the
examples below, a sandwich ELISA wherein the monoclonal in
the hybridoma supernatant is bound both to antigen and to
15   an antihuman constant region can be employed. Conversely,
hybridomas that secrete antibodies which are
immunoreactive with antispecies antibodies directed to the
species of the immunized animal can be discarded. After
the appropriate hybridomas are selected, the desired
20   antibodies can be recovered, again using conventional
techniques. They can be prepared in quantity by culturing
the immortalized B cells using conventional methods,
either *in vitro*, or *in vivo* to produce ascites fluid.
Purification of the resulting monoclonal antibody
25   preparations is less burdensome than in the case of serum
since each immortalized colony will secrete only a single
type of antibody. In any event, standard purification
techniques to isolate the antibody from other proteins in
the culture medium can be employed.
30   As an alternative to obtaining human
immunoglobulins directly from the culture of immortalized

B cells derived from the animal, the immortalized cells can be used as a source of rearranged heavy chain and light chain loci for subsequent expression and/or genetic manipulation. Isolation of genes from such antibody-

5 producing cells is straightforward since high levels of the appropriate mRNAs are available for production of a cDNA library. The recovered rearranged loci can be manipulated as desired. For example, the constant region can be exchanged for that of a different isotype or that

10 of a human antibody, as described above, or eliminated altogether. The variable regions can be linked to encode single chain F_V regions. Multiple F_V regions can be linked to confer binding ability to more than one target or chimeric heavy and light chain combinations can be

15 employed. Once the genetic material is available, design of analogs as described above which retain their ability to bind the desired target, as well as their human characteristics, is straightforward.

Once the appropriate genetic material is

20 obtained and, if desired, modified to encode an analog, the coding sequences including those that encode, at a minimum, the variable regions of the human heavy and light chain can be inserted into expression systems contained on vectors which can be transfected into standard recombinant

25 host cells. As described below, a variety of such host cells may be used; for efficient processing, however, mammalian cells are preferred. Typical mammalian cell lines useful for this purpose include CHO cells, 293 cells, or NSO-GS cells.

30 The production of the antibody or analog is then undertaken by culturing the modified recombinant host

under culture conditions appropriate for the growth of the
host cells and the expression of the coding sequences.
The antibodies are then recovered from the culture. The
expression systems are preferably designed to include
5 signal peptides so that the resulting antibodies are
secreted into the medium; however, intracellular
production is also possible.

In addition to deliberate design of modified
forms of the immunoglobulin genes to produce analogs,
10 advantage can be taken of phage display techniques to
provide libraries containing a repertoire of antibodies
with varying affinities for the desired antigen. For
production of such repertoires, it is unnecessary to
immortalize the B cells from the immunized animal; rather
15 the primary B cells can be used directly as a source of
DNA. The mixture of cDNAs obtained from B cells, e.g.,
derived from spleens, is used to prepare an expression
library, for example, a phage display library transfected
into *E. coli*. The resulting cells are tested for
20 immunoreactivity to the desired antigen. Techniques for
the identification of high affinity human antibodies from
such libraries are described by Griffiths, A.D., *et al.*,
EMBO J (1994) 13:3245-3260; by Nissim, A., *et al. ibid,*
692-698, and by Griffiths, A.D., *et al., ibid*, 725-734.
25 Ultimately, clones from the library are identified which
produce binding affinities of a desired magnitude for the
antigen, and the DNA encoding the product responsible for
such binding is recovered and manipulated for standard
recombinant expression. Phage display libraries may also
30 be constructed using previously manipulated nucleotide
sequences and screened in similar fashion. In general,

the cDNAs encoding heavy and light chain are independently
supplied or are linked to form F_v analogs for production
in the phage library.

The phage library is thus screened for the
antibodies with highest affinity for the antigen and the
genetic material recovered from the appropriate clone.
Further rounds of screening can increase the affinity of
the original antibody isolated. The manipulations
described above for recombinant production of the antibody
or modification to form a desired analog can then be
employed.

As above, the modified or unmodified rearranged
loci are manipulated using standard recombinant techniques
by constructing expression systems operable in a desired
host cell, such as, typically, a Chinese hamster ovary
cell, and the desired immunoglobulin or analog is produced
using standard recombinant expression techniques, and
recovered and purified using conventional methods.

The application of the foregoing processes to
antibody production has enabled the preparation of human
immunospecific reagents with respect to antigens for which
human antibodies have not heretofore been available. The
immunoglobulins that result from the above-described
methods and the analogs made possible thereby, provide
novel compositions for use in analysis, diagnosis,
research, and therapy. The particular use will, of
course, depend on the immunoglobulin or analog prepared.
In general, the compositions of the invention will have
utilities similar to those ascribable to nonhuman
antibodies directed against the same antigen. Such
utilities include, for example, use as a affinity ligands

for purification, as reagents in immunoassays, as components of immunoconjugates, and as therapeutic agents for appropriate indications.

Particularly in the case of therapeutic agents 5 or diagnostic agents for use *in vivo*, it is highly advantageous to employ antibodies or their analogs with fully human characteristics. These reagents avoid the undesired immune responses engendered by antibodies or analogs which have characteristics marking them as 10 originating from non-human species. Other attempts to "humanize" antibodies do not result in reagents with fully human characteristics. For example, chimeric antibodies with murine variable regions and human constant regions are easily prepared, but, of course, retain murine 15 characteristics in the variable regions. Even the much more difficult procedure of "humanizing" the variable regions by manipulating the genes encoding the amino acid sequences that form the framework regions does not provide the desired result since the CDRs, typically of nonhuman 20 origin, cannot be manipulated without destroying immunospecificity. Thus, the methods of the present invention provide, for the first time, immunoglobulins that are fully human or analogs which contain immunospecific regions with fully human characteristics.

25 There are large numbers of antigens for which human antibodies and their human analogs would be made available by the methods of the invention. These include the following as a nonlimiting set:

leukocyte markers, such as CD2, CD3, CD4, CD5, 30 CD6, CD7, CD8, CD11a,b,c, CD13, CD14, CD18, CD19, CD20, CD22, CD23, CD27 and its ligand, CD28 and its ligands

B7.1, B7.2, B7.3, CD29 and its ligand, CD30 and its ligand, CD40 and its ligand gp39, CD44, CD45 and isoforms, CDw52 (Campath antigen), CD56, CD58, CD69, CD72, CTLA-4, LFA-1 and TCR

histocompatibility antigens, such as MHC class I or II, the Lewis Y antigens, SLex, SLey, SLea, and SLeb;

integrins, such as VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, and LFA-1;

adhesion molecules, such as Mac-1 and p150,95;

selectins, such as L-selectin, P-selectin, and E-selectin and their counterreceptors VCAM-1, ICAM-1, ICAM-2, and LFA-3;

interleukins, such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-11, IL-12, IL-13, IL-14; and IL-15;

interleukin receptors, such as IL-1R, IL-2R, IL-4R, IL-5R, IL-6R, IL-7R, IL-8R, IL-10R, IL-11R, IL-12R, IL-13R, IL-14R, and IL-15R;

chemokines, such as PF4, RANTES, MIP1α, MCP1, NAP-2, Groα, Groβ, and IL-8;

growth factors, such as TNFalpha, TGFbeta, TSH, VEGF/VPF, PTHrP, EGF family, FGF, PDGF family, endothelin, and gastrin releasing peptide (GRP);

growth factor receptors, such as TNFalphaR, RGFbetaR, TSHR, VEGFR/VPFR, FGFR, EGFR, PTHrPR, PDGFR family, EPO-R, GCSF-R and other hematopoietic receptors;

interferon receptors, such as IFNαR, IFNβR, and IFNγR;

Igs and their receptors, such as IgE, FceRI, and FCeRII;

tumor antigens, such as her2-neu, mucin, CEA and endosialin;

allergens, such as house dust mite antigen, lol p1 (grass) antigens, and urushiol;

5   viral proteins, such as CMV glycoproteins B, H, and gCIII, HIV-1 envelope glycoproteins, RSV envelope glycoproteins, HSV envelope glycoproteins, EBV envelope glycoproteins, VZV envelope glycoproteins, HPV envelope glycoproteins, Hepatitis family surface antigens;

10   toxins, such as pseudomonas endotoxin and osteopontin/uropontin, snake venom, and bee venom;

blood factors, such as complement C3b, complement C5a, complement C5b-9, Rh factor, fibrinogen, fibrin, and myelin associated growth inhibitor;

15   enzymes, such as cholesterol ester transfer protein, membrane bound matrix metalloproteases, and glutamic acid decarboxylase (GAD); and

miscellaneous antigens including ganglioside GD3, ganglioside GM2, LMP1, LMP2, eosinophil major basic 20 protein, eosinophil cationic protein, pANCA, Amadori protein, Type IV collagen, glycated lipids, γ-interferon, A7, P-glycoprotein and Fas (AFO-1) and oxidized-LDL.

Particularly preferred immunoglobulins and analogs are those immunospecific with respect to human 25 IL-6, human IL-8, human TNFα, human CD4, human L-selectin, and human gp39. Human antibodies against IL-8 are particularly useful in preventing tumor metastasis and inflammatory states such as asthma. Antibodies and analogs immunoreactive with human TNFα and human IL-6 are 30 useful in treating cachexia and septic shock as well as autoimmune disease. Antibodies and analogs immunoreactive

with gp39 or with L-selectin are also effective in
treating or preventing autoimmune disease. In addition,
anti-gp39 is helpful in treating graft versus host
disease, in preventing organ transplant rejection, and in
5 treating glomerulonephritis. Antibodies and analogs
against L-selectin are useful in treating ischemia
associated with reperfusion injury.

Typical autoimmune diseases which can be treated
using the above-mentioned antibodies and analogs include
10 systemic lupus erythematosus, rheumatoid arthritis,
psoriasis, Sjogren's, scleroderma, mixed connective tissue
disease, dermatomyositis, polymyositis, Reiter's syndrome,
Behcet's disease, Type 1 diabetes, Hashimoto's
thyroiditis, Grave's disease, multiple sclerosis,
15 myasthenia gravis and pemphigus.

The examples below are intended to illustrate
but not to limit the invention.

In these examples, mice, designated "xenomice",
are used for initial immunizations. A detailed
20 description of such xenomice is found in the above
referenced PCT application WO 94/02602. Immunization
protocols appropriate to each antigen are described in the
specific examples below. The sera of the immunized
xenomice (or the supernatants from immortalized B cells)
25 were titrated for antigen specific human antibodies in
each case using a standard ELISA format. In this format,
the antigen used for immunization was immobilized onto
wells of microtiter plates. The plates were washed and
blocked and the sera (or supernatants) were added as
30 serial dilutions for 1-2 hours of incubation. After
washing, bound antibody having human characteristics was

detected by adding the appropriate antispecies Ig (typically antihuman kappa or antihuman μ) conjugated to horseradish peroxidase (HRP) for one hour. In some cases, the bound antibodies were tested for murine

5   characteristics using antimurine antibodies, typically antimurine γ. After again washing, the chromogenic reagent o-phenylene diamine (OPD) substrate and hydrogen peroxide were added and the plates were read 30 minutes later at 492 nm using a microplate reader.

10   Unless otherwise noted, the antigen was coated using plate coating buffer (0.1 M carbonate buffer, pH 9.6); the assay blocking buffer used was 0.5% BSA, 0.1% Tween 20 and 0.01% Thimerosal in PBS; the substrate buffer used in color development was citric acid 7.14 g/l:

15   dibasic sodium phosphate 17.96 g/l; the developing solution (made immediately before use) was 10 ml substrate buffer, 10 mg OPD, plus 5 ml hydrogen peroxide; the stop solution (used to stop color development) was 2 M sulfuric acid. The wash solution was 0.05% Tween 20 in PBS.

20   Example 1
                    Human Antibodies Against Human IL-6
          Three to 5 xenomice aged 8-20 weeks were age-
matched and immunized intraperitoneally with 50 μg human
IL-6 emulsified in complete Freund's adjuvant for primary

25   immunization and in incomplete Freund's adjuvant for subsequent injections. The mice received 6 injections 2-3 weeks apart. Serum titers were determined after the second dose and following each dose thereafter. Bleeds were performed 6-7 days after injections from the

30   retrobulbar plexus. The blood was allowed to clot at room

temperature for about 2 hours and then incubated at 4°C for at least 2 hours before separating and collecting the sera.

ELISAs were conducted as described above by

5   applying 100 µl per well of recombinant human IL-6 at 2 mg/ml in coating buffer. Plates were then incubated at 4°C overnight or at 37°C for 2 hours and then washed three times in washing buffer. Addition of 100 µl/well blocking buffer was followed by incubation at room temperature for

10   2 hours, and an additional 3 washes.

Then, 50 µl/well of diluted serum samples (and positive and negative controls) were added to the plates. Plates were then incubated at room temperature for 2 hours and again washed 3 times.

15   After washing, 100 µl per well of either HRP- mouse antihuman IgM at 1/2,000 or HRP-mouse antihuman kappa at 1/2,000, diluted in blocking buffer were added. After a 1 hour incubation at room temperature, the plates were washed 3 times and developed with OPD substrate for

20   10-25 minutes. 50 µl/well of stop solution were then added and the results read on an ELISA plate reader at 492 nm. The dilution curves resulting from the titration of serum from xenomouse A40-7 after 6 injections are shown in Figure 1. The data in Figure 1 show production of anti-

25   IL-6 immunoreactive with antihuman kappa and antihuman µ detectable at serum dilutions above 1:1,000.

Example 2
Human Antibodies Against Human IL-8

Immunization and serum preparation were as described in Example 1 as except that human recombinant
5   IL-8 was used as an immunogen.

ELISA assays were performed with respect to the recovered serum, also exactly as described in Example 1, except that the ELISA plates were initially coated using 100 μl/well of recombinant human IL-8 at 0.5 mg/ml in the
10 coating buffer. The results obtained for various serum dilutions from xenomouse A260-5 after 6 injections are shown in Figure 2. Human anti-IL-8 reactivity was again shown at serum dilutions having concentrations higher than that represented by a 1:1,000 dilution.

15   ## Example 3
Human Antibodies Against Human TNFα

Immunization and serum preparation were conducted as described in Example 1 except that human recombinant TNFα was substituted for human IL-6. ELISAs
20 were conducted as described in Example 1 except that the initial coating of the ELISA plate employed 100 μl/well recombinant human TNFα at 1 mg/ml in coating buffer.

The dilution curves for serum from xenomouse A210-8 after 6 injections obtained are shown in Figure 3.
25 Again significant titers of human anti-TNFα reactivity were shown.

Example 4

Human Antibodies Against Human CD4

The human CD4 antigen was prepared as a surface protein on transfected recombinant cells as follows:

5   Human CD4 zeta (F15 LTR) as described in Roberts, et al., Blood (1994) 84:2878 was introduced into the rat basophil leukemic cell line RBL-2H3, described by Callan, M., et al., Proc Natl Acad Sci USA (1993) 90:10454 using the kat high efficiency transduction system described by Finer, et

10 al., Blood (1994) 83:43. Briefly, RBL-2H3 cells at 10^6 cells per well were cultured in 750 ml DMEM^{low} + 20% FBS (Gibco) and 16 μg/ml polybrene with an equal volume of proviral supernatant for 2 hours at 37°C, 5% CO_2. One ml of medium was removed and 750 μl of infection medium and

15 retroviral supernatant were added to each well and the cultures incubated overnight. The cells were washed and expanded in DMEM^{low} + 10% FBS until sufficient cells were available for sorting. The CD4-zeta transduced RBL-2H3 cells were sorted using the FACSTAR plus (Becton

20 Dickinson). The cells were stained for human CD4 with a mouse antihuman CD4-PE antibody and the top 2-3% expressing cells were selected.

Immunizations were conducted as described in Example 1 using 10 x 10^6 cells per mouse except that the

25 primary injection was subcutaneous at the base of the neck. The mice received 6 injections 2-3 weeks apart. Serum was prepared and analyzed by ELISA as described in Example 1 except that the initial coating of the ELISA plate utilized 100 μl per well of recombinant soluble CD4

30 at 2 mg/ml of coating buffer. The titration curve for serum from xenomouse A207-1 after 6 injections is shown in

Figure 4. Titers of human anti-CD4 reactivity were shown at concentrations representing greater than those at 1:1,000 dilution.

Example 5

5 Human Antibodies Against Human L-selectin

The antigen was prepared as a surface displayed protein in C51 cells, a high expressing clone derived by transfecting the mouse pre-B cell 300.19 with LAM-1 cDNA (LAM-1 is the gene encoding L-selectin) (Tedder, et al., J

10 Immunol (1990) 144:532) or with similarly transfected CHO cells. The transfected cells were sorted using fluorescent activated cell sorting using anti-Leu-8 antibody as label.

The C51 and the transfected CHO cells were grown

15 in DME 4.5 g/l glucose with 10% FCS and 1 mg/ml G418 in 100 mm dishes. Negative control cells, 3T3-P317 (transfected with gag/pol/env genes of Moloney virus) were grown in the same medium without G418.

Primary immunization was by injection

20 subcutaneously at the base of the neck; subsequent injections were intraperitoneal. 70-100 million C51 or transfected CHO cells were used per injection for a total of five injections 2-3 weeks apart.

Sera were collected as described in Example 1

25 and analyzed by ELISA in a protocol similar to that set forth in Example 1.

For the ELISA, the transfected cells were plated into 96 well plates and cell monolayers grown for 1-2 days depending on cell number and used for ELISA when

30 confluent. The cells were fixed by first washing with

cold 1 x PBS and then fixing solution (5% glacial acetic acid, 95% ethanol) was added. The plates were incubated at -25°C for 5 minutes and can be stored at this temperature if sealed with plate sealers.

5   The ELISA is begun by bringing the plates to room temperature, flicking to remove fixing solution and washing 5 times with DMEM medium containing 10% FCS at 200 μl per well.

The wells were treated with various serum
10   dilutions or with positive or negative controls. Positive control wells contained murine IgG1 to human L-selectin.

The wells were incubated for 45 minutes and monolayer integrity was checked under a microscope. The wells were then incubated with either HRP-antimouse IgG
15   (1/1000) or with the antihuman kappa or antihuman μ conjugates described in Example 1. The plates were then washed with 1% BSA/PBS and again with PBS and monolayer integrity was checked. The plates were developed, stopped, and read as described above. The results for
20   serum from xenomouse A303-3 are shown in Figs. 5 and 6; human antibodies both to L-selectin and control 3T3 cells were obtained. However, the serum titers are higher for the L-selectin-expressing cells as compared to parental 3T3 cells. These results show that xenomouse A303-3
25   produces antibodies specific for L-selectin with human μ heavy chain regions and/or human kappa light chains.

ELISAs were also performed using as the immobilized antigen a fusion protein consisting of the extracellular domain of human L-selectin fused to the
30   constant domain of human IgG$_1$ (Guo, et al., <u>Cell Immunol</u> (1994) <u>154</u>:202). The L-selectin fusion protein was made

by transient transfection of human 293 cells using calcium phosphate transfection (Wigler, M., Cell (1979) 16:777). Serum preparation was performed as described in Example 1. ELISAs were conducted essentially as in Example 1, except
5   that the initial coating of the ELISA plate employed 100 μl transfected 293 cell culture supernatant containing the L-selectin-Ig fusion protein. Detection employed HRP-mouse antihuman kappa and HRP-goat antimouse IgG.

       Figure 7 shows the results from xenomouse
10   A195-2; antibodies specific for L-selectin having human kappa light chains and/or murine heavy chain γ regions are present in the serum.

       The antisera obtained from the immunized xenomice were also tested for staining of human
15   neutrophils. Human neutrophils were prepared as follows: peripheral blood was collected from normal volunteers with 100 units/ml heparin. About 3.5 ml blood was layered over an equal volume of One-step Polymorph Gradient (Accurate Chemical, Westbury, NY) and spun for 30 minutes at 450 x G
20   at 20°C. The neutrophil fraction was removed and washed twice in DPBS/2% FBS.

       The neutrophils were then stained with either:

       (1) antiserum from xenomouse A195-2 immunized with C51 cells (expressing L-selectin);
25   (2) as a positive control, mouse monoclonal antibody LAM1-3 (against L-selectin); and

       (3) as negative control, antiserum from a xenomouse immunized with cells expressing human gp39.

       The stained, washed neutrophils were analyzed by
30   FACS. The results for antiserum from xenomouse A195-2 are shown in Figures 8 and 9.

These results show the presence of antibodies in
immunized xenomouse serum which contain fully human
variable regions immunoreactive with L-selectin. The
negative control antiserum from mice immunized with gp39
5   does not contain antibodies reactive against human
neutrophils. Serum from A195-2 (immunized with
L-selectin-expressing cells) contains antibodies binding
to human neutrophils detectable with a goat antimouse IgG
(Figure 8), which immunoreacts with heavy chain protein
10 composed of fully human variable regions and mouse γ
constant regions. Staining with anti L-selectin xenomouse
antisera detected with a mouse monoclonal antibody against
human kappa is shown in Figure 9, showing the presence of
fully human kappa light chain.
15   As explained above, these antibodies containing
human variable regions are readily convertible to fully
human antibodies. For example, using hybridomas secreting
these antibodies, the cDNAs encoding them can be obtained.
By amplifying the genes encoding human V regions using
20 primers containing restriction enzyme recognition sites
and cloning them into plasmids containing the coding
sequences for human constant regions as described by
Queen, et al., Proc Natl Acad Sci (1989) 86:10029, genes
encoding the fully human antibodies can be obtained for
25 recombinant production.

Example 6

Human Antibodies Against Human gp39

gp39 (the ligand for CD40) is expressed on
activated human CD4+ T cells. The sera of xenomice
30 immunized with recombinant gp39 according to this example

contained antibodies immunospecific for gp39 with fully
human variable regions; the sera contained fully human IgM
antibodies and chimeric IgG antibodies containing human
variable regions and murine constant heavy chain γ region.

5   The antigen consisted of stable transfectants of
300.19 cells or of CHO cells expressing gp39 cDNA cloned
into the mammalian expression vector P1K1.HUgp39/IRES NEO
as shown in Figure 10. CHO cells were split 1:10 prior to
transfection in DMEM 4.5 g/l glucose, 10% FBS, 2 mM
10   glutamine, MEM, NEAA supplemented with additional glycine,
hypoxanthine and thymidine. The cells were cotransfected
with the gp39 vector at 9 μg/10 cm plate (6 X 10^5 cells)
and the DHFR expressing vector pSV2DHFRs (Subranani et al.
Mol Cell Biol (1981) 9:854) at 1 μg/10 cm plate using
15   calcium phosphate transfection. 24 hours later the cells
were split 1:10 into the original medium containing G418
at 0.6 mg/ml. Cells producing gp39 were sorted by FACS
using an anti-gp39 antibody.

        Mice grouped as described in Example 1 were
20   immunized with 300.19 cells expressing gp39 using a
primary immunization subcutaneously at the base of the
neck and with secondary intraperitoneal injections every
2-3 weeks. Sera were harvested as described in Example 1
for the ELISA assay. The ELISA procedure was conducted
25   substantially as set forth in Example 1; the microtiter
plates were coated with CHOD-gp39 cells grown in a 100 mm
dish in DMEM, 4.5 g/l glucose, 10% FCS, 4 mM glutamine,
and nonessential amino acid (NEAA) solution for MEM
(100X). On the day preceding the ELISA assay, the cells
30   were trypsinized and plated into 96-well filtration plates
at 10^5 cells/200 μl well and incubated at 37°C overnight.

The positive controls were mouse antihuman gp39; negative controls were antisera from mice immunized with an antigen other than gp39. 50 μl of sample were used for each assay. The remainder of the assay is as described in
5 Example 1.

The dilution curves for the sera obtained after 4 injections from mice immunized with gp39 expressed on CHO cells are shown in Figure 11. As shown, the sera contained antihuman gp39 immunospecificity which is
10 detectable with human kappa and human μ HRP-coupled antibodies.

In addition, the sera were tested for their ability to react with activated human T cells included in PBMC using FACS analysis. To prepare the PBMC, human
15 peripheral blood was collected from normal volunteers with the addition of 100 unit/ml heparin. PBMC were isolated over Ficoll gradient and activated with 3 μg/ml PHA, 1 μg/ml PMA in IMDM plus 10% FBS plus 25 μM 2-mercaptoethanol for 4 hours. After washing, the PBMC
20 were stained with mouse Mab against human CD4 labeled with FITC to permit separation of activated human T cells from unactivated cells.

The activated CD4+ and CD4- T cells were then analyzed by FACS using staining with either:
25   1)   antiserum from a Xenomouse immunized with 300.19 cells producing gp39;
          2)   a positive control mouse Mab directed against α-CD40L (human gp39); and
          3)   a negative control antiserum from a
30 Xenomouse immunized with TNF.

The detecting antibody in the FACS analysis was goat antimouse IgG (PE). The results are shown in Figure 12.

As shown in Figure 12A, CD4+ (R2) and CD4- (R3)
5 cells were separated prior to FACS analysis. Panel B shows the results for CD4+ cells and shows that sera from mice immunized with gp39 (labeled A247-4 in the figure) reacted with these activated PBMC; panel C shows that these sera did not react with CD4- cells. These
10 antibodies carried immune heavy chain γ constant regions. The results of panels B and C confirm that the TNF-injected xenomouse did not make antibodies against activated human T cells.

Example 7
15 ## Preparation of High-Affinity Human Mabs
Against Tetanus Toxin

The antibodies prepared in this example were secreted by hybridomas obtained by immortalizing B cells from xenomice immunized with tetanus toxin. The
20 immunization protocol was similar to that set forth in Example 1 using 50 μg tetanus toxin emulsified in complete Freund's adjuvant for intraperitoneal primary immunization followed by subsequent intraperitoneal injections with antigen incorporated into incomplete Freund's adjuvant.
25 The mice received a total of 4 injections 2-3 weeks apart.

After acceptable serum titers of antitetanus toxinC (anti-TTC) were obtained, a final dose of antigen in PBS was given 4 days before the animals were sacrificed and the spleens harvested for fusion.

The spleen cells were fused with myeloma cells
P3X63-Ag8.653 as described by Galfre, G. and Milstein, C.
Methods in Enzymology (1981) 73:3-46.

After fusion the cells were resuspended in DMEM,
5   15% FCS, containing HAT supplemented with glutamine,
pen/strep for culture at 37°C and 10% CO_2. The cells were
plated in microtiter trays and maintained in HAT-
supplemented medium for two weeks before transfer to HT-
supplemented media. Supernatants from wells containing
10   hybridomas were collected for a primary screen using an
ELISA.

The ELISA was conducted as described in Example
1 wherein the antigen coating consisted of 100 μl/well of
tetanus toxin C (TTC) protein at 2 mg/ml in coating
15   buffer, followed by incubation at 4°C overnight or at 37°C
for two hours. In the primary ELISA, HRP-conjugated goat
antimouse IgG at 1/2000 was used in addition to HRP mouse
antihuman IgM as described in Example 1. Two hybridomas
that secreted anti-TTC according to the ELISA assay, clone
20   D5.1 and clone K4.1 were used for further analysis.

As shown in Figure 13, clone D5.1 secretes fully
human anti-TTC which is detectable using HRP-conjugated
antihuman μ and HRP-conjugated antihuman kappa. Figure 14
shows that clone K4.1 secretes anti-TTC which is
25   immunoreactive with antimurine γ and antihuman kappa HRP-
conjugated antibodies. Thus, clone K4.1 provides anti-TTC
with human variable regions and a murine constant heavy
chain γ region.

The antibodies secreted by D5.1 and K4.1 did not
30   immunoreact in ELISAs using TNFα, IL-6, or IL-8 as
immobilized antigen under conditions where positive

controls (sera from xenomice immunized with TNFα, IL-6 and IL-8 respectively) showed positive ELISA results.

The affinity of the monoclonal antibodies secreted by K4.1 for TTC antigen was determined using
5 commercially available reagents and instrumentation. BIAcore Instrument, CM5 sensor chips, surfactant P20 and the amine coupling kit were purchased from Pharmacia Biosensor (Piscataway, NJ). TTC was immobilized at two levels of antigen density on the surface of the sensor
10 chips according to the manufacturer's instructions. Briefly, after washing and equilibrating the instrument with buffer containing surfactant, the surfaces were activated and the TCC was immobilized.

For high antigen density, the surface was
15 activated with 35 μl of equal volumes 0.1 M NHS and 0.1 M EDC injected across the surface followed by 30 μl of TTC fragment at 100 μg/ml in 10 mM sodium acetate buffer pH 5.0. The surface was blocked by injecting 35 μl 1 M ethanolamine and washed to remove noncovalently bound TCC
20 using 5 μl 0.1 M HCl. The entire immobilization procedure was conducted with a continuous flow of buffer at 5 μl/min. This results in about 7500-8500 response units (RU) of TTC per chip. (1000 RU corresponds to about 1 ng of protein per mm^2.)

25 For chips with low antigen density, the procedure utilizes 15 μl rather than 30 μl of TTC, resulting in chips containing 550-950 RU.

Chips could be regenerated after use in single determinations by injecting 10 μl formal or $MgCl_2$.
30 The chips are used to determine binding affinities by determining k_a and k_b (the association and

dissociation rate constants) for the antibody with respect
to the immobilized TTC. The association rate constant is
measured over six minutes at a flow rate of 5 μl/min. at
different concentrations of K4.1 Mab in the range of
5   2.16 nm-69.33 nm. The dissociation rate constant is
measured at a constant buffer flow rate of 5 μl/min after
completion of the antibody injection. The raw data are
graphed in Figure 15 and the calculated results are shown
in Table 1.

Table 1

Kinetic Constants of K4.1 Measured Using the BIAcore on Two Different Surface

Immobilized tetanus toxinC	K4.1 conc. range nM	Association rate $ka(10^5 \ M^{-1}s^{-1})$	Dissociation rate $kd(10^5 s^{-1})$	Binding constant $KA(M^{-1})=ka/kd$	Dis c KD
5931 RU	4.3 - 34.7	6.47 ± 1.05	4.02 ± 1.42	$1.6 \ X \ 10^{10}$	0.6
868 RU	4.3 - 34.7	7.19 ± 2.18	2.02 ± 1.01	$3.5 \ x \ 10^{10}$	0.2

840228.APP

As shown, the K4.1 antibody has a binding
constant for TTC somewhat larger than 10^{10} 1/mol.

The complete nucleotide sequence of the cDNAs
encoding the heavy and light chains of the K4.1 and D5.1
5   monoclonals were determined as shown in Figures 16-19.
PolyA mRNA was isolated from about 10^6 hybridoma cells and
used to generate cDNA using random hexamers as primers.
Portions of the product were amplified by PCR using the
appropriate primers.

10   Both cell lines were known to provide human
kappa light chains; for PCR amplification of light chain
encoding cDNA, the primers used were HKP1
(5'-CTCTGTGACACTCTCCTGGGAGTT-3')
for priming from the constant region terminus and two
15   oligos, used in equal amounts to prime from the variable
segments: B3 (5'-CCACCATCAACTGCAAGTCCAGCCA-3') and B2/B1
(5'-GAAACGACACTCACGCAGTCTCCAGC-3').

For amplification of the heavy chain from K4.1
(which contains the murine γ1 constant region), the
20   primers were MG-24Vi for the human variable regions:
5'-CAGGTGCAGCTGGAGCAGTCiGG-3' which, with inosine as shown
recognizes the human variable regions V_{H1-2}, V_{H1-3}, V_{H4} and
V_{H6}, and from the constant region MG-25 i.e.,
5'-GCACACCGCTGGACAGGGATCCAiAGTTTC-3', which, containing
25   inosine as shown recognizes murine γ1, γ2A, γ2B, and γ3.

For amplification of the heavy chain of the
antibody derived from D5.1 (which contains the human μ
constant region), MG-24VI was used to prime from the
variable and μP1 (5'-TTTTCTTTGTTGCCGTTGGGGTGC-3') was used
30   to prime from the constant region terminus.

Turning first to the results shown in Figure 16 representing the heavy chain of the Mab secreted by K4.1, the sequence shows the presence of the human variable segment VH6, the human diversity region DN1, and the human
5 joining segment JH4 linked to the murine $\gamma 1$ constant region. Nine base-pair mutations from the published germline sequence were present in the variable region, two of them within CDR2. One mutation was observed in the D segment. Three nongermline nucleotide additions were
10 present in the D_H-J_H junction.

Referring to Figure 17 which shows the light chain of the K4.1 antibody, analysis shows the presence of the human kappa variable region B3 and joining region JK4. Eight nucleotides are missing from B3 at the V_K-J_K
15 junction and four mutations were found in the variable region. Five nongermline nucleotide additions were present at the V_K-J_K junction.

Referring now to Figure 18 which sets forth the sequence for the heavy chain of the antibody secreted by
20 clone D5.1, this shows the heavy chain is comprised of the human variable fragment VH6, the human diversity region DN1 and the human joining segment JH4 linked to the human μ constant region. There were two base-pair mutations from the germline sequence in the variable region, neither
25 within the CDRs. Two additional mutations were in the D segment and six nongermline nucleotide additions were present at the D_H-J_H junction.

Finally, referring to Figure 19 which presents the light chain of the antibody secreted by D5.1, the
30 human kappa variable region B3 and human kappa joining region JK3 are shown. There are nine base-pair

differences from the germline sequences, three falling
within CDR1.